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Hepatitis C Virus Infection: The Role of Pexophagy

A thesis submitted in partial satisfaction of the
requirements for the degree Master of Science

in

Biology

by

Shereen Nourollahi

Committee in charge:

Professor Aleem Siddiqui, Chair
Professor Randolph Hampton, Co-Chair
Professor James Kadonaga
Professor Deborah Spector

2013

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Co-Chair

Chair

University of California, San Diego

2013

Dedication

This research project and thesis is dedicated to all my family, friends, and mentors around me who I am tremendously blessed to have supporting me in pursuing my dreams and ambitions.

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Abstract Of The Thesis

Hepatitis C Virus Infection: The Role of Pexophagy

by

Shereen Nourollahi

Master of Science in Biology

University of California, San Diego, 2013

Professor Aleem Siddiqui, Chair
Professor Randolph Hampton, Co-Chair

Cellular peroxisomes function in detoxification of reactive oxygen species (ROS), β -oxidation of lipids, and innate immune defense, cooperatively with the mitochondria. Hepatitis C virus perturbs these cellular functions and the organelles involved to promote a lipid and ROS rich cellular microenvironment, favorable for hepatitis C virus (HCV) proliferation. Several groups have also shown that HCV utilizes the host autophagy machinery for its replication. In addition, the induction of autophagy by HCV also serves to restrain the cellular innate immune defense mechanisms. In this study, we investigated HCV mediated induction of selective autophagic degradation of peroxisomes, or pexophagy. Our conclusions, using a series of standard experimental procedures, show that peroxisomes undergo a complete autophagy under HCV infection.

I:

Introduction

Hepatitis Virus

Hepatitis is a liver associated disease that has been noticed by humanity since ancient times.¹ It translates from Greek as “liver inflammation”, with symptoms such as jaundice, which is a yellowing of the skin, eyes and body fluids.² There are six common hepatitis viruses, HAV, HBV, HCV, HDV (a delta agent), HEV, and HGV. These viruses are unrelated to each other at the molecular, organizational, and biological levels. HBV and HCV cause chronic disease and hepatocellular carcinoma.

Although the indications of liver disease including jaundice have been noticed for thousands of years, Hepatitis viruses were not discovered until the middle of the 20th century. The first virus discovered was hepatitis B in the 1960s, and Hepatitis A virus was identified in 1974.³ Today, the combined chronic viral hepatitis associated infections affect over 500 million people worldwide.⁴

Hepatitis C Virus

Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma worldwide.⁵ It is estimated that the virus is no younger than one thousand years old, based on its diversity of genotypes.⁶ Approximately 3% of the world population is currently infected with HCV.⁷ The prevalence is higher (up to 15%) in some countries in Africa and Asia, and highest (>15%) in Egypt. The most common mode of transmission in the United States is direct blood contact with affected individuals, by sharing syringes and other drug-related instruments among people who inject drugs.⁸

The course of chronic hepatitis C varies. Symptoms are usually nonspecific, and include general sickness and fatigue, with fewer than 20% of patients experiencing anything noticeable. An estimated 20% to 30% of patients with chronic hepatitis C go on to develop cirrhosis.⁹ Liver cancer is a usual effect from the devastating harms of hepatitis, and liver transplantation is quite often suggested for patients.¹⁰ However, recurrent HCV infection occurs in >90% infected liver transplant recipients.

HCV Therapies

At the current time, therapies that exist with a dedicated proven response against HCV-induced liver disease are non-existent. The absence of genetic proofreading by the viral RNA polymerase allows HCV to have a high mutation rate, which allows the variants or quasispecies to exist within a patient.¹¹ This property of viral polymerase is also an impediment in the development of vaccine.

Current therapy includes a combination of interferon-alpha (IFN-alpha) and ribavirin (RBV).¹² However, HCV genotypes respond to treatment differently. Genotype 1a /1b which is prevalent in the US and Europe responds to IFN poorly.

Recently, two “direct-acting antiviral” (DAA) agents, Telaprevir and Boreceprvir, have been approved by the FDA, with impressive results. Both are HCV protease inhibitors, but the emergence of HCV mutants against these inhibitors poses a major problem. Thus, novel antiviral therapeutics are still needed to arrest chronic hepatitis worldwide.

HCV Genomics and Molecular Background

HCV is classified in the Hepacivirus genus within the Flaviviridae family.¹³ Shared structural and virological characteristics are seen throughout members of the Flaviviridae family. The HCV genome is a positive strand RNA about 9.6 kb in length. It encodes an open reading frame (ORF), which is translated into a 3,000-amino acid polyprotein and contains three structural (core, E1, E2) and seven non-structural (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins.^{14,15} The RNA genome also contains a relatively long untranslated region (UTR) of 342 nucleotides at the 5' end and about 230 nucleotides at the 3' end. With no polyA tail, both 5' and 3' ends fold into complex stem-loop structures (Figure 1).¹⁶

The 5' UTR region of HCV contains a highly conserved structured domain.¹⁷⁻²⁰ Within this region an internal ribosome entry site (IRES) resides, consisting of four domains, I-IV.¹⁸ The uncapped HCV RNA genome initiates translation via its IRES element by first recruiting the 40s ribosomal subunit to form a pre-initiation complex without the canonical translation initiation factors (eIFs). Translation of the viral RNA by the IRES mechanism occurs on the rough endoplasmic reticulum.¹⁹ HCV replicates in the ER-modified membranous structures, which remain to be characterized, in the form of ribonucleoprotein complexes (RNP), and viral titer in a given patient can reach up to 1×10^{12} virions a day.²⁰

HCV Life Cycle

The HCV viral entry mechanism is a complex multistep process mediated by several entry factors located on the cell surface, including the tetraspanin CD81, the

scavenger receptor class B member I (SR-BI), Claudin 1 (CLDN1), Occludin (OCLN), and low-density lipoprotein receptor (LDLR). The virus is usually associated with lipoproteins in patient's sera and the association with the lipoproteins determines its infectivity. The internal ribosome entry site in the 5' region of the HCV RNA genome facilitates translation. The viral RNA encoded polypeptide of about 3,000 amino acids that is cleaved by cellular and viral proteases into 10 matured viral proteins (Figure 1). The structural proteins core, E1, E2, constitute the viral particle whereas seven nonstructural protein (NS) proteins p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B participate in the replication of viral RNA and the assembly of viral particles. Several reports implicate that lipid droplets, the neutral lipid storage cellular organelles, play a key role in HCV life cycle.²¹ The core protein localizes to the surface of lipid droplets and then subsequently facilitates recruitment of other NS proteins to the lipid droplets. Inhibition of core association to lipid droplets disrupts the assembly of viral particles.^{22,23,24} Although the precise mechanism involved in secretion or egress of the matured viral particle is currently unknown, several reports suggest that HCV hitches a ride with the very low-density lipoprotein for secretion via the Golgi-secretory pathway.

Changes in cellular physiology associated with HCV Infection

HCV relies on host lipid metabolism for the viral RNA replication, viral particle assembly and secretion. Hence HCV modulates the hepatocyte lipid metabolism to facilitate enrichment of intracellular lipids, which serve essential function in multiple aspects of HCV life cycle. HCV promotes *de novo* lipogenesis and hinders the catabolic

breakdown (b-oxidation of fatty acids) and lipid export. HCV infection is also associated with endoplasmic reticulum stress and oxidative stress triggered by high levels of reactive oxygen species (ROS). Many of the HCV proteins associate with ER resulting in enhanced ER load and ER stress. ER-stress associated with HCV infection results in depletion of ER- Ca^{2+} stores.³⁰ Additionally depletion of ER- Ca^{2+} disrupts the protein folding capacity of promoting accumulation of unfolded proteins in the ER and ER stress.²⁵ The leaked Ca^{2+} is subsequently taken up by the mitochondria closely located near the ER through the mitochondrial calcium uniporter resulting in high mitochondrial calcium levels that initiate a cascade of events culminating in mitochondrial membrane depolarization followed by mitochondria dysfunction and damage.³¹ The reactive oxygen species (ROS) produced during HCV infection also promote mitochondrial dysfunction and damage, which in addition also produce ROS. Normally, reactive oxygen species will be maintained at levels that are counterbalanced by cellular antioxidants, which prevent ROS from contributing to any dangerous cell damage.²⁶ However, when this critical balance is disrupted, such as by HCV infection, ROS accumulates in cells causing oxidative stress. Increased oxidative protein folding, oxidative damaged to lipids, DNA, perturbed redox balance and reduced oxidative phosphorylation has been shown in HCV infected cells. High levels of ROS in a cell can cause oxidative damage to DNA initiating DNA mutagenesis and cancer growth. Patients chronically infected with HCV often develop hepatocellular carcinoma. Livers from chronic hepatitis C patients exhibit high ROS and reduced antioxidants levels.²⁷ The metabolic and physiologic changes associated with HCV infection promote a lipid and reactive oxygen species rich microenvironment, which is essential for HCV replication complexes.^{28,29,30}

HCV virus utilizes autophagic machinery for viral RNA replication, and recent studies demonstrate that autophagy vesicles may probably serve as viral RNA replication platforms. Like many other viruses, HCV infection promotes autophagy to facilitate viral propagation. ER-stress and high levels of ROS associated with HCV infection have been shown to promote induction of autophagy.³¹ Another advantage associated with induction of autophagy is to promote degradation of damaged cellular organelles like mitochondria via organ-specific autophagy to promote rapid turnover of cellular organelles. This sustains cellular homeostasis and prevents lethal cellular damage facilitating cellular viability and as a consequence persistent viral infection. Autophagy is also known to negatively regulate the cellular innate immune response, which helps HCV to evade the innate immune defense mechanisms. Since mitochondria are also involved in innate immune processes the mitochondrial dysfunction associated with HCV infection also facilitates HCV evade the cellular innate immunity. In addition the HCV NS3–4A protease cleaves the mitochondrial antiviral signaling protein (MAVS) resulting in its dissociation from the mitochondria subsequently contributing in prevention of mitochondrial antiviral signaling during HCV infection.^{32, 33, 34}

Autophagy within a Cell

Macro-autophagy is a cellular process that enables a cell to maintain “housekeeping” duties, such as recycling damaged or unneeded organelles, proteins, removing intracellular pathogens, and in nutrient supplementation during conditions of starvation.³⁵ The process is initiated by the formation of an autophagophore, or double-layered membrane, around the cargo to be degraded within a cell. Phagophore formation

is initiated by the processing of microtubule-associated protein, light chain 3 (LC3B) (Figure 4). LC3B is expressed in most cell types as a full-length cytosolic protein that, upon induction of autophagy, is proteolytically cleaved by autophagy related protein 4, or Atg4, a cysteine protease, to generate LC3B-I. Atg7 then activates the carboxyterminal glycine exposed by the Atg4 in an ATP-dependent manner. LC3B-II is then produced by the conjugating of phosphatidylethanolamine molecule to LC3B-I by Atg3, a carrier protein. The synthesis and conversion of LC3B-I to LC3B-II is greatly increased during autophagic process, which enables its use as a suitable marker to evaluate induction of autophagy in cells.³⁶ This new molecule, LC3B-II, is dispersed throughout the newly forming autophagosome around the content to be degraded, aiding in the nucleation of the phagophore membranes and also in determination of the cargo to be degraded.

After the autophagosome vesicle completely engulfs the content to be degraded, it must fuse with the lysosomes to facilitate the degradation of the associated cargo (Figure 4). The fusion of autophagosomes with lysosomes generates the autolysosome.³⁷

This part of the autophagy process is less well understood, yet studies have shown it requires specific proteins to recognize and merge the two vesicular organelles together. Two of the proteins are a GTP-bound G protein, Rab7, and the Presenilin protein.^{38,39,40} The cellular cytoskeleton, along with lysosomal associated membrane proteins 1 and 2 (Lamp-1 and Lamp-2) within the lysosome, are also important factors in assisting the merging of the autophagosome with the lysosome.^{41,42}

After merging and the completed autophagolysosome is developed, internal hydrolases within the lysosomal compartment act upon the cargo that is now present for degradation, and the disintegrated matter is then either taken to the edge of the cell

membrane where it is expelled, or the content which may have valuable energy or cellular building blocks is released back into the cytosol for reuptake in useful ways.⁴³

Why Peroxisome Specific Autophagy?

Recent studies have shown that like other viruses, HCV induces bulk autophagy to facilitate viral replication. Targeted deletion of autophagic machinery or suppressing LC3 lipidation is known to affect HCV replication.⁴⁴ Our laboratory is currently working on evaluating organelle-specific autophagy during HCV infection. HCV promotes mitochondrial dysfunction and damage leading to accumulation of damaged mitochondria, which need to be rapidly removed to maintain cellular viability and viral persistence. Mitophagy is the mitochondrial-specific autophagic process to facilitate the rapid turnover of damaged mitochondria. Drs. Kim and Syed in the laboratory identified the mitochondrial translocation of Parkin, a key mediator of mitophagy, by confocal microscopy and its presence in the highly purified mitochondrial preparations isolated from HCV-infected cells. Further, our lab demonstrated that Parkin's translocation to mitochondria is followed by engulfment of damaged mitochondria in autophagosomes (mitophagosomes) and their subsequently delivery to the lysosomes. We also show that mitophagy is functionally relevant to HCV replication. The knockdown of Parkin and PINK1, two key mediators of mitophagy, significantly decreased HCV replication. Our lab confirmed that HCV stimulates both Parkin and PINK1 expression at the transcriptional level. We also identified that HCV-mediated decrease of mitochondrial complex I activity is restored upon inhibition of mitophagy or silencing Parkin.⁴⁵

Together, these results provide a role of mitophagy in the maintenance of cellular homeostasis in HCV infection and implicate a functional role of damaged mitochondria in viral replication processes and liver disease pathogenesis.⁴⁶

Mitochondria and peroxisomes both have been identified to have analogous functions in lipid metabolism involving β -oxidation of fatty acids and in innate immune responses. Both the organelles are also pivotal for maintenance of ROS levels in the cell. However, peroxisomes have been assumed to be completely metabolic-focused organelles while mitochondria are known to be involved in antiviral signaling and apoptosis.⁴⁷ Yet even though both organelles have several functional and morphological differences, studies confirm that peroxisomes and mitochondria cooperate with each other in cellular homeostasis.^{48, 49}

Peroxisomes are responsible for counteracting many of the harmful events that cells face when HCV overtakes their system. They are major players in removing the reactive oxygen species in cells. Peroxisomes can also be seen as the “clean up” organelles of foreign invaders in addition, making them essential to focus on when it comes to viral invasion. In addition, HCV is known to suppress the β -oxidation of fatty acids and peroxisomes also play a major role in β -oxidation of fatty acids in addition to the mitochondria, hence we envisaged that like mitochondria-specific autophagy, HCV may also promote peroxisomal-specific autophagy to sustain a lipid and oxidant rich intracellular environment within the infected hepatocytes to facilitate its proliferation and pathogenesis. Hence on this basis we chose to investigate the involvement of peroxisomal-specific autophagy or pexophagy in HCV infection. Much thanks goes to

Dr. Suresh Subramani's laboratory for providing us the tools to make our conclusions possible.

II:

Materials and Methods

Reagents and Plasmids:

Rabbit polyclonal antibody against catalase, mouse monoclonal against LAMP2 was purchased from Abcam. Mouse monoclonal anti-HCV Core antibody was purchased from Affinity bioreagents. LC3B and b-actin antibody was purchased from Cell Signaling Technology. Guinea pig antibody against PMP70 was a gift from Prof. Suresh Subramani, University of California, San Diego. The high titer HCV plasmid p JC1 and mouse monoclonal 9E10 antibody against NS5A, were gifts of Dr. C. Rice, Rockefeller University, New York. Human monoclonal antibody E2 was kind gift of Dr. Mansun Law, The Scripps Research Institute, La Jolla, California. The plasmids pAT003 expressing mRFP-EGFP fused with the peroxisomal localization sequence RSKL, pLA59 encoding the human peroxisomal trans-2enoyl-CoA-Reductase fused with EGFP and pTF-LC3 or mRFP-EGFP-LC3 plasmid encoding LC3 protein fused to RFP and GFP to study autophagic flux were kind gifts from Prof. Suresh Subramani, University of California, San Diego. pEGFP-LC3 plasmid was a kind gift of Dr. Tamotsu Yoshimori, National Institute of Genetics, Japan

Cell Culture

Human hepatoma cell lines Huh7 and Huh7.5.1 were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% Pen Strep, and 1 mM minimal essential medium with nonessential amino acids (Invitrogen, Carlsbad, CA). Huh7.5.1 cells were a kind gift of Dr. Frank Chisari, The Scripps Research Institute, La Jolla, CA.

HCV infection

The plasmid pJC1 encoding the genome of highly infectious genotype 2a chimera JC1 was linearized by XbaI digestion, and subsequently subjected for RNA synthesis using a RiboMAX Large Scale RNA production system-T7 (Promega, Madison, WI). 10 µg of extracted HCV RNA was used for electroporation using a Gene Pulser Xcell electroporation system in a 4mm cuvette at 260 volts for 25 milliseconds (Bio-Rad, Hercules, CA). 3-5 day post electroporation the culture supernatants containing the infectious HCV virus particles were collected and clarified at 4000 rpm. The clarified culture supernatants were stored in -80C until further use. Naïve Huh7 cells were infected with the cell culture derived (HCVcc) chimeric HCV–JC1 virus at 0.1 MOI. After 3 days post infection the cells were used for immunofluorescence analysis. For all the other experiments the HCV infected Huh7 cells were propagated as needed.

Transfection

Uninfected or HCV infected Huh7 cells were transfected with respective plasmids as indicated with Mirus Trans-IT LT1 transfection reagent (Mirus Bio, Madison, WI) according to the manufacturers instruction. 48h post transfection, the cells were fixed in 4% PFA and processed for immunostaining as described below.

Western Blot Analysis:

Uninfected and HCV infected Huh7 cells grown in 100mm culture dishes upon reaching confluency were washed in ice-cold PBS, and scraped in 1ml ice-cold PBS. The

cell pelleted by centrifugation for 5 mins at 4000 rpm at 4C. The cell pellet was lysed in ice with 300 μ l of RIPA lysis buffer (Tris 50mM, NaCl 150 mM, SDS 0.1 %, Na.Deoxycholate 0.5 %, Triton X 100, NP40 1%, and PMSF). The lysate was incubated on ice for 30 mins and then subjected to brief sonication followed by centrifugation at $12,000 \times g$ for 20 min. The clarified lysates were aliquoted and kept frozen in -80C until further use. The protein concentration in the lysates estimated using the BioRads protein assay kit according to the manufacturer's instruction. About 10-20 mg total protein from each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred onto nitrocellulose membrane at 15 volts overnight at 4C. The nitrocellulose membranes were then blocked in 5% milk blocking solution (with 0.2% Tween 20, 0.05% Sodium Azide) for 1h at room temperature. Primary antibodies were usually prepared in PBS containing 3%BSA or as directed by the manufacturer at dilutions recommended for Western blotting. Respective primary antibodies were used to probe the membrane overnight at 4C with gentle rocking. After 3 washes with PBS containing 0.1% Tween 20 (PBST) the membranes were probed with respective HRP-conjugated secondary antibodies in PBS for 1h at room temperature followed by 3x washes in PBST. After the final wash the Western blots were imaged using HRP chemiluminescent substrate in Kodak Chemiluminescent Image Station.

Immunofluorescence microscopy

Uninfected and HCV infected Huh7 cells transfected with respective plasmids were grown on glass coverslips. 48h post transcription the cells were washed twice in PBS and fixed in 4% paraformaldehyde for 20 m at room temperature. Fixed cells were

permeabilized in 50 mM digitonin for 10mins. The permeabilized cells were washed 3x for 5 mins each in PBS and then blocked for 1h at room temperature in PBS containing 1% BSA and 0.1% Tween 20. The cells were then probed with respective primary antibodies diluted in blocking buffer for overnight at 4C. After 3x washes for 5mins each in PBS the cells were incubated in respective Donkey Alexa-Fluor secondary antibodies (Invitrogen, Carlsbad, CA) diluted in blocking buffer for 1h at room temperature. After 3x washes in PBS for 5 mins each the nuclei were counterstained by DAPI (4',6'-diamidino-2-phenylindole) (Invitrogen, Carlsbad, CA) and mounted onto glass slides using the Prolong Gold Antifade medium (Invitrogen, Carlsbad, CA). Images were acquired with Olympus FluoView 1000 confocal microscope with a 60 or 100X oil objective.

III:
Results

Autophagy during HCV infection

To determine if HCV induced autophagy, human hepatoma cells, Huh7, infected with cell culture derived HCV virus (HCVcc) at 0.1 MOI were seeded on glass coverslips after 3-days post infection and subjected to transfection with pEGFP-LC3 plasmid that encodes LC3-GFP fusion protein. 48h post transfection the cells were fixed in 4% PFA and HCV infected cells immunostained with anti-HCV core antibody as described in materials and methods (Figure 4). pEGFP-LC3 is commonly used to monitor induction of autophagy. In normal cells EGFP-LC3 is diffused all over the cytoplasm, however in cells-induced for autophagy EGFP-LC3 localizes in distinct cytoplasmic puncta that represent autophagosomes. HCV-infected cells displayed distinct EGFP-LC3 puncta (Figure 4). In contrast, EGFP-LC3 in uninfected cells exhibited diffused localization all throughout the cytoplasm (Figure 4). We then performed Western blotting for LC3B protein in whole cell lysates obtained from HCV-infected and uninfected Huh7 cells (Figure 5). Upon induction of autophagy LC3B-I undergoes lipidation and is converted to LC3B-II that migrates differentially to LC3B-I in protein gel electrophoresis. Western blot analysis with anti-LC3-B antibody identifies both the LC3B-I and -II bands. Western blot analysis of LC3B in uninfected and HCV-infected cells clearly displayed a dramatic increase in the proportion of LC3B-II in HCV infected cells in comparison to uninfected cells (Figure 5). Together, these observations reaffirm the previously published reports that HCV infection triggers autophagy as evidenced by formation of EGFP-LC3 puncta (Figure 4) and LC3B-I lipidation to LC3B-II (Figure 5).

Induction of Peroxisome-Specific Autophagy by HCV

To determine if HCV induced peroxisomal-specific autophagy or pexophagy, the HCV infected Huh7 cells transfected with pEGFP-LC3 plasmid were simultaneously immunostained for HCV core and catalase, a peroxisomal protein (Figure 6). Immunofluorescence microscopy showed that the EGFP-LC3 puncta in HCV infected cells distinctly overlapped with catalase at multiple locations within the cell (Figure 6 see the white arrows in LC3GFP+Catalase merged panel). The immunofluorescence images also revealed that all the EGFP-LC3 puncta did not merge or colocalize with catalase suggesting that at a given time point only a fraction of total cellular peroxisomes are undergoing pexophagy. In contrast the uninfected cells hardly display any overlap or colocalization between EGFP-LC3 puncta and catalase (Figure 6, see uninfected cells). Overall these observations suggest that in HCV infected Huh7 cells a fraction of cellular peroxisomes are undergoing pexophagy.

Peroxisome levels decline during HCV infection

To determine if HCV induces peroxisomes degradation we monitored peroxisome levels in HCV-infected and uninfected Huh7 cells. For this purpose uninfected and HCV infected Huh7 cells (at 0.1 MOI) were propagated for about 9 days post infection and cells collected at several time points (days 1, 2, 3, 7, and 9) as indicated. Whole cell lysates prepared from these cells were subjected to Western blot analysis of catalase and peroxisomal matrix protein 70 (PMP70) (Figures 7 & 8). The Western blot analysis clearly suggests that as the HCV infection spreads there is a concomitant decline in the total level of peroxisomes, which is determined by the decline in the level of catalase and PMP70 (Figures 7 & 8). Western blot analysis of HCV core protein was used to

determine HCV infection and b-actin was used as internal protein loading control to show that equal amount of protein was used in all the respective lanes. For better inference of the obtained results we also performed densitometric analysis of respective catalase and PMP70 bands. The graphics displaying the band intensity normalized against b-actin values are incorporated below the respective Western blots of catalase and PMP70 in Figure 6 & 7. In this experiment we used HCV infected cells from different time points (days 1, 2, 3, 7, and 9) to determine if the decline in peroxisome levels proceeds along with the HCV infection and correlates to the viral load. The results clearly suggest that as the HCV infection progresses there is a corresponding decline in peroxisomes levels suggesting that it is not a transient phenomenon which occurs early during infection and subsequently stabilizes (Figure 6 & 7). The direct correlation between viral load and peroxisomal levels is more clearly evident in Western blots of PMP70 (Figure 7). Together these results confirm that HCV infection promotes a decline in total cellular peroxisome levels and it remains to be determined if this decline is a consequence of pexophagy or reduced peroxisome biogenesis.

We then performed immunofluorescence analysis of catalase (Figure 9) in HCV-infected and uninfected Huh7 cells to determine variation in the level of peroxisomes at cellular level. In correlation to the Western blot analysis of peroxisomes levels the immunofluorescence analysis also revealed that the HCV-infected cells displayed less number of peroxisomes (visualized by catalase immunostaining) in comparison to the uninfected cell (Figure 9). The inset in figure 9 shows the HCV envelope protein E2 staining to show that cell in consideration is infected. The graphically representation of relative fluorescence intensity of catalase determined by Image J analysis in HCV vs

uninfected cells shown in figure 9 clearly suggests that there is over 2-fold decline in peroxisomes levels in HCV-infected cells compared to uninfected cells. In order to determine if the decline in peroxisome levels during HCV infection is not associated with reduced peroxisome biogenesis but due to enhanced degradation via pexophagy we used the plasmid pLA59 that encodes an EGFP tagged peroxisomal protein, trans-2enoyl-CoA-Reductase under the control of CMV promoter of the plasmid (Figure 10). Therefore any significant reduction in the expression of GFP fused trans-2enoyl-CoA-reductase in HCV infected Huh7 cells compared to uninfected cells would directly imply that the reduced expression is a consequence of enhanced peroxisomal degradation and not a cause of reduced gene expression. The immunofluorescence analysis of pLA59 transfected control and HCV-infected cells clearly demonstrates reduced GFP fluorescence in HCV-infected Huh7 cells in comparison to uninfected cells. Since the GFP expression is under the CMV promoter, a decline in GFP expression suggests enhanced peroxisomal degradation in HCV-infected cells compared to uninfected cells (Figure 10).

Does HCV promote complete Autophagy?

We observed that peroxisomes are associated with LC3-GFP puncta suggesting that they are probable engulfed by the phagophore. However, earlier studies have claimed that HCV induces incomplete autophagy which does not correlate with our observation that the total peroxisomal levels decline in HCV infected cells. Hence using the pTF-LC3 plasmid we proceeded to determine if in HCV-infected cells autophagy is completely executed. pTF-LC3 encodes GFP and RFP tagged LC3 protein. As shown in

the figure 11 this dual-tag system allows us to tag specific proteins to follow their path through autophagy and will assist in determining if the particular organelle or protein is subjected to complete autophagy. Usually a protein double tagged with GFP and RFP displays yellow color (merge of green and red fluorescence) when in the cytosol. Upon delivery to the lysosomes red fluorescence is observed due to the relatively high stability of RFP in lysosomal compartment compared to GFP. We transfected HCV-infected Huh7 cells with pTF-LC3 and 48h after transfection the cells were fixed in 4% PFA and immunostained for HCV core protein (Figure 12, see the blue panel). Our results show that the RFP+GFP merge panel displayed a fraction of distinct red spots (Figure 12, see the white arrows in the merged panel) within the predominant yellow signal (Figure 12). This suggests that the GFP and RFP are coexpressed and as expected in the cytosol display yellow color due to the merge of green and red fluorescence. However upon delivery of the GFP-RFP-LC3 to the lysosomes GFP being unstable compared to RFP in the acidic lysosomal environment there is shift of the yellow signal towards red fluorescence from RFP (Figure 11 & 12). This suggests that in HCV infection complete autophagy occurs resulting in the fusion of autophagosomes to lysosomes resulting in the formation of autophagolysosomes and subsequent degradation of the deliver cargo.

Are Peroxisomes Going to the Lysosomes?

As we confirmed that HCV triggers complete autophagy determined we wanted to determine if the peroxisomes/pexophagosmes fuse with the lysosomes resulting in the formation of pexophagolysosomes. Initially we performed immunofluorescence analysis of HCV-infected Huh7 cells immunostained for catalase (peroxisomes marker) and lysosomes associated membrane protein 2 (Lamp2), a lysosome marker (Figure 13).

Immunofluorescence analysis revealed that in both the uninfected and HCV-infected Huh7 cells peroxisomes (identified by catalase staining, Green) merge/colocalize with lysosomes (identified by Lamp2 staining, Red) (Figure 13, see the green+red merged panel) signifying that in both the cells peroxisomes are undergoing complete pexophagy. HCV core staining is shown in blue. Our observations suggest that although both the uninfected and HCV-infected cells display merge of peroxisomes and lysosomes, the infected cells display higher level of colocalization compared to uninfected cells (Figure 13, see the white arrows in the zoomed section). This suggests that in uninfected Huh7 cells physiological/basal level of pexophagy occurs however in HCV-infected cells pexophagy occurs at a higher rate. To further consolidate our observations we used the innovative technique of using GFP-RFP double-tagged peroxisomal protein to monitor the pexophagy flux and also to determine if complete pexophagy occurs (Figure 11). We transfected HCV-infected Huh7 cells with pAT003 plasmid, which encodes GFP-RFP in fusion with a C-terminal serine-lysine-leucine SKL sequence making it localize to the peroxisomes. As explained in the model (Figure 11) RFP is more stable than GFP in the acidic compartments of the lysosomes, hence we observe a red signal when the double-tagged protein/organelle is delivered to the lysosomes. The immunofluorescence analysis of the HCV-infected cells transfected with pAT003 clearly display a strong yellow signal from the combination of green and red fluorescence (Figure 14, see merged panel). However we also observe distinct red spots indicating that a fraction of peroxisomes are delivered to the lysosomes (Figure 14, see white arrows in the merged panel).

Autophagy Inhibitors

Finally, we wanted to see if inhibition of pexophagy restores the peroxisomes levels. Currently no mammalian proteins playing a role in pexophagy have been identified yet. Hence we attempted to block pexophagy using general autophagy inhibitors like 3-methyladenine (3-MA) inhibits the phagophore formation involving LC3 and Bafilomycin, (BafA1) a vacuolar ATPase inhibitor that suppresses fusion between autophagosomes and lysosomes. Uninfected and HCV infected cells transfected with pLA59 which encode a GFP-tag peroxisomal protein and 24h post transfection the cells were treated with 1mM 3-MA and 50 nM Bafilomycin for 12h. The cells were fixed in 4% PFA and cells immunostained for LAMP2. We imaged the cells with confocal microscopy and saw that 3-MA and Bafilomycin treatment partially rescued peroxisomal levels in infected cells, and Bafilomycin almost fully restored peroxisome localized GFP expression to physiological levels (Figure 15).

We also performed a Western blot of the same cells, and the results coincided with those of the microscopy. The control lane showed a decrease in catalase in the HCV infected cells, as predicted. Yet in 3-MA and Bafilomycin treated cells, catalase levels were restored as in uninfected Huh7 cells (Figure 16).

IV:
Discussion

HCV causes chronic hepatitis. HCV infected cells are maintained and there is no apparent cell lysis or apoptosis. Autophagy has emerged as an important regulatory event that sustain persistent phenotype of infected hepatocytes. Previous studies have shown that HCV induces bulk autophagy. This work focuses on selective autophagy of peroxisomes. Pexophagy induced by HCV is evidenced by presence of LC3 puncta in peroxisomes and subsequent migration to lysosomes. The initial punctae formation of LC3-GFP in HCV infected cells suggests that higher than normal autophagy is occurring. Subsequent analysis through Western blotting supports bulk autophagy (Figure 5). Further investigations in which we show the presence of LC3 puncta in association with peroxisome clearly supports the view that peroxisomes are involved in the process and indeed HCV induces pexophagy.

Furthermore our data shows that there is a significant decrease in peroxisomal levels in cells that are infected with HCV (Figures 7-9). Using standard and novel methods of determining the path that peroxisomes take within an infected cell, we determined that HCV does induce a complete pexophagy, in contrast to other studies that have been previously reported on bulk autophagy. Next, we demonstrate the engulfment of peroxisomes in the lysosomal compartment. This is evidence by the presence of LC3 puncta in association with lysosomes (Figure 13). GFP-RFP double-tagged peroxisomal protein, an innovative strategy developed in Subramani's laboratory, confirms for us that complete pexophagy is in fact occurring.

Rescue of peroxisomal levels by autophagy inhibitors suggests that pexophagy in HCV infected cells is a dynamic and occurs at higher levels in comparison to uninfected cells.

The effect of HCV on pexophagy highlights the role of oxidative stress in HCV life cycle and liver disease pathogenesis. HCV-mediated modulation of pexophagy signifies the virus's effort to curb the antioxidant signaling and maintain an intracellular oxidative environment. Thus these studies open new avenues to liver disease pathogenesis associated with HCV infection. Future work should characterize in depth the mechanisms of pexophagy by HCV and if individual proteins are capable of inducing pexophagy and how it may benefit viral infectious process such as replication, morphogenesis, and egress. Clinical liver biopsies should be examined for pexophagy.

V:
Figures

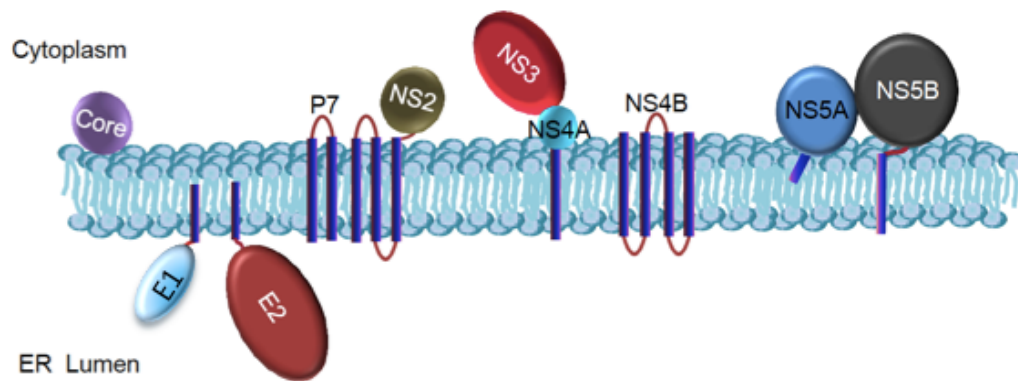
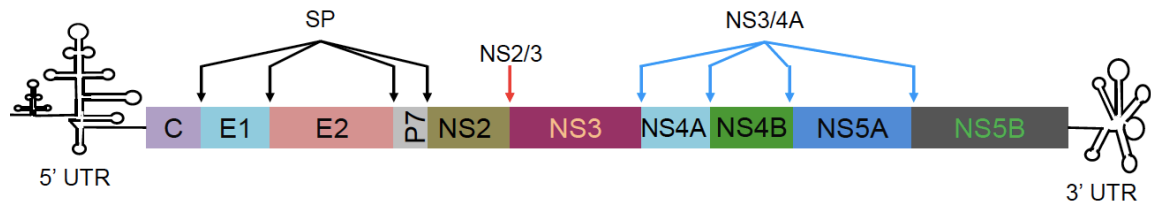


Figure 1: Schematic representation of HCV genome (above) and proteins tethered to the ER (below). The 9.6-kb positive-strand RNA genome is shown with 5'- and 3'-untranslated regions (UTR). IRES-mediated translation yields a polyprotein precursor that is processed into the mature structural and non-structural proteins by host and viral proteases as indicated. The structural and nonstructural viral proteins in association with ER membrane and their orientation is shown.

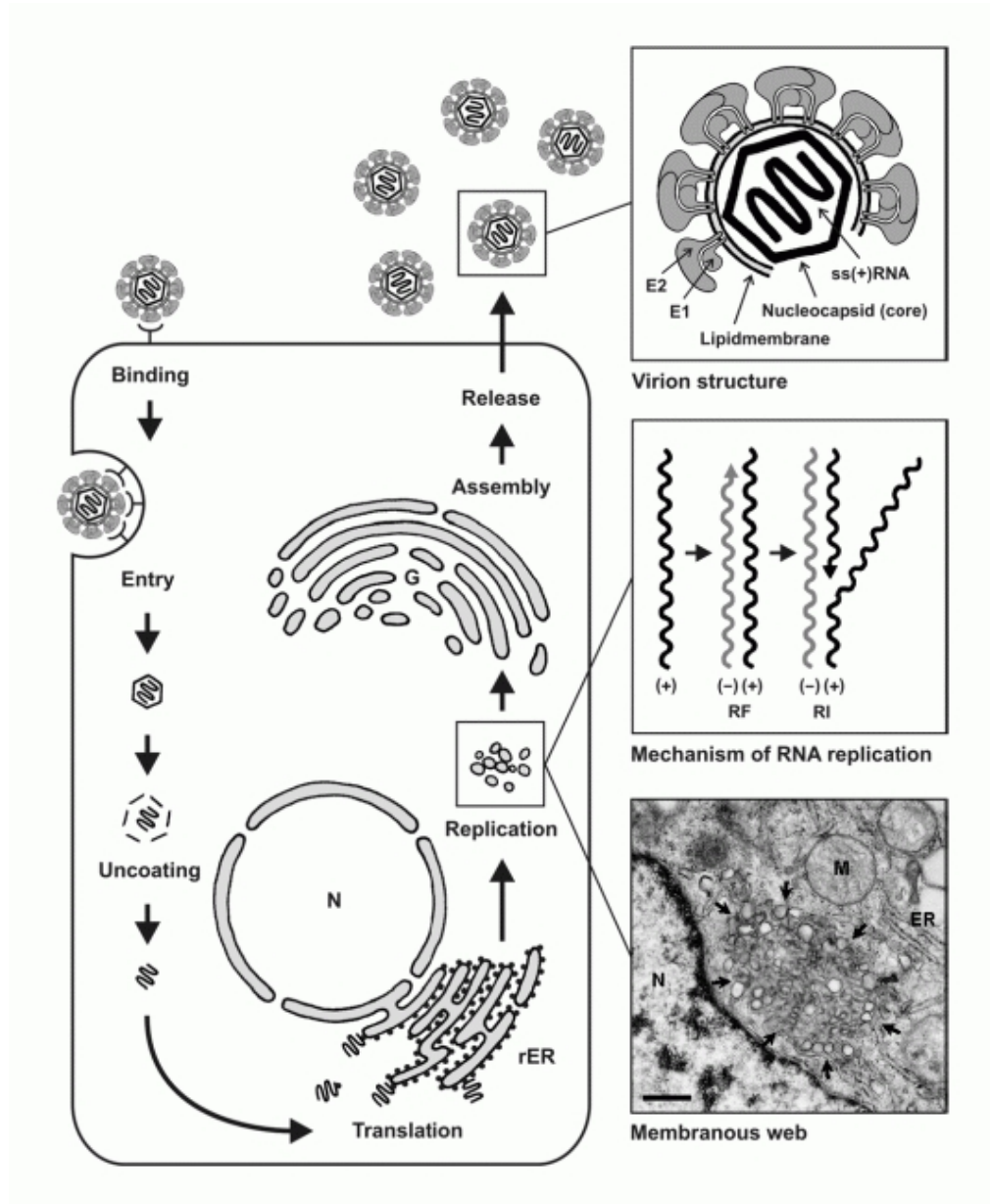


Figure 2: HCV life cycle. The virus gains entry into the cells utilizing the various cell surface receptors via receptor mediated endocytosis. Post internalization the virus is uncoated in acidic compartments and the viral genome released. The HCV genome undergoes cap-independent translation mediated by the IRES to yield a 3000 aa polyprotein. Host and viral proteases further process the polyprotein to generate 10 mature viral protein. HCV-NS5B, an RNA dependent RNA polymerase mediated HCV RNA replication on the replication complex assembled on ER-derived membranous structures known as “membranous web”. Virus particle assembly occurs on the lipid droplets that serve as platforms for virus particle morphogenesis. The mature virus particles hitch a ride on the very low-density lipoprotein particles via the Golgi-secertory pathway N: nucleus; ER: endoplasmic reticulum; M: mitochondria. (Image Bartenschlager et al., 2004)

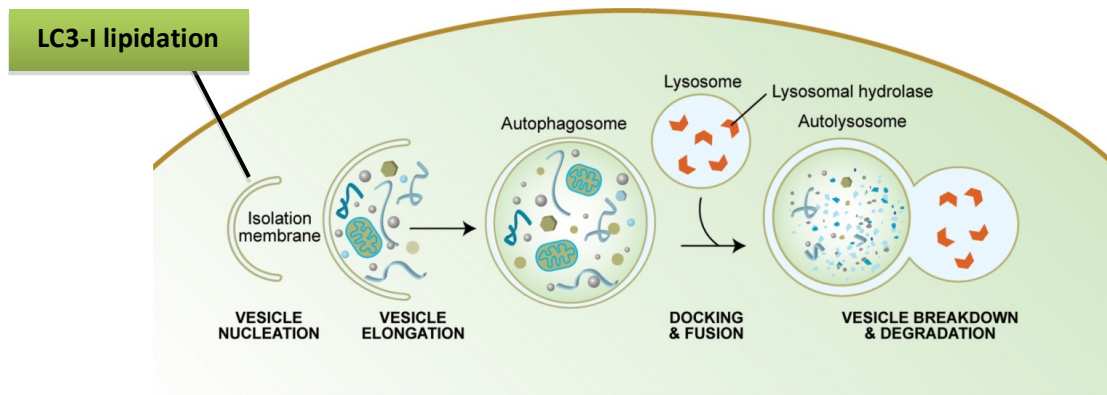


Figure 3: Model depicting the various stages in the autophagy pathway. An autophagosome is first developed by membrane formation in the cytosol around cellular matter to be degraded, recruited by LC3-I lipidation to LC3-II. The mature autophagosome then fuses with a lysosome to generate an autolysosome. In the autolysosome, the cargo is then degraded by hydrolases. (Image: Meléndez, A. and Levine, B. Autophagy in *C. elegans*)

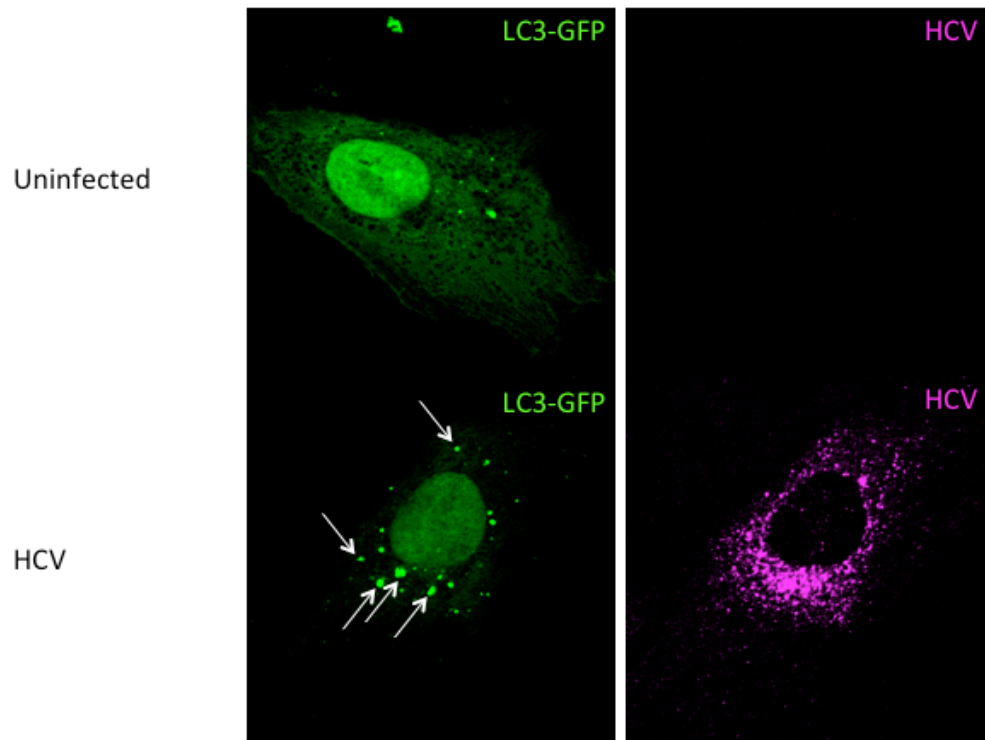


Figure 4: HCV triggers the formation of LC3-GFP puncta. Induction of Autophagy is observed by transfection of HCV-infected and uninfected Huh7 cells with LC3-GFP plasmid. LC3-GFP staining is shown in green and HCV core protein staining is shown in pink. White arrow mark the LC3-GFP puncta observed in HCV infected cells. Confocal images reveal that HCV infected cells display LC3 puncta in contrast to diffused cellular distribution observed in uninfected cells.

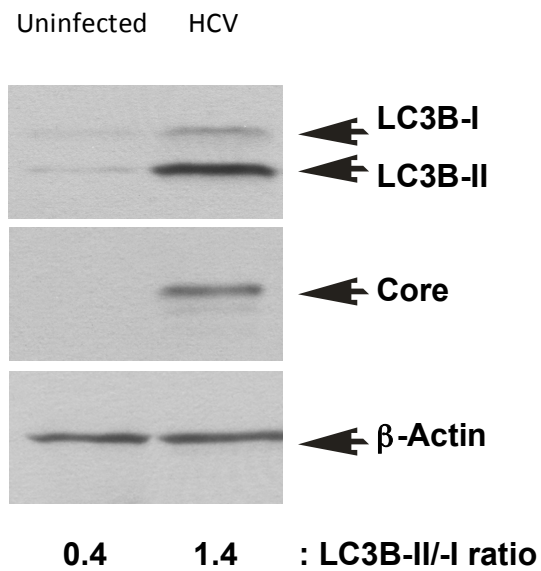


Figure 5. HCV triggers the formation of LC3 lipidation. LC3 lipidation was evaluated by Western blot analysis in HCV infected and uninfected Huh7 cells. HCV core is used as infection marker and beta-actin as internal loading control. The ratio of LCB-II/I is shown.

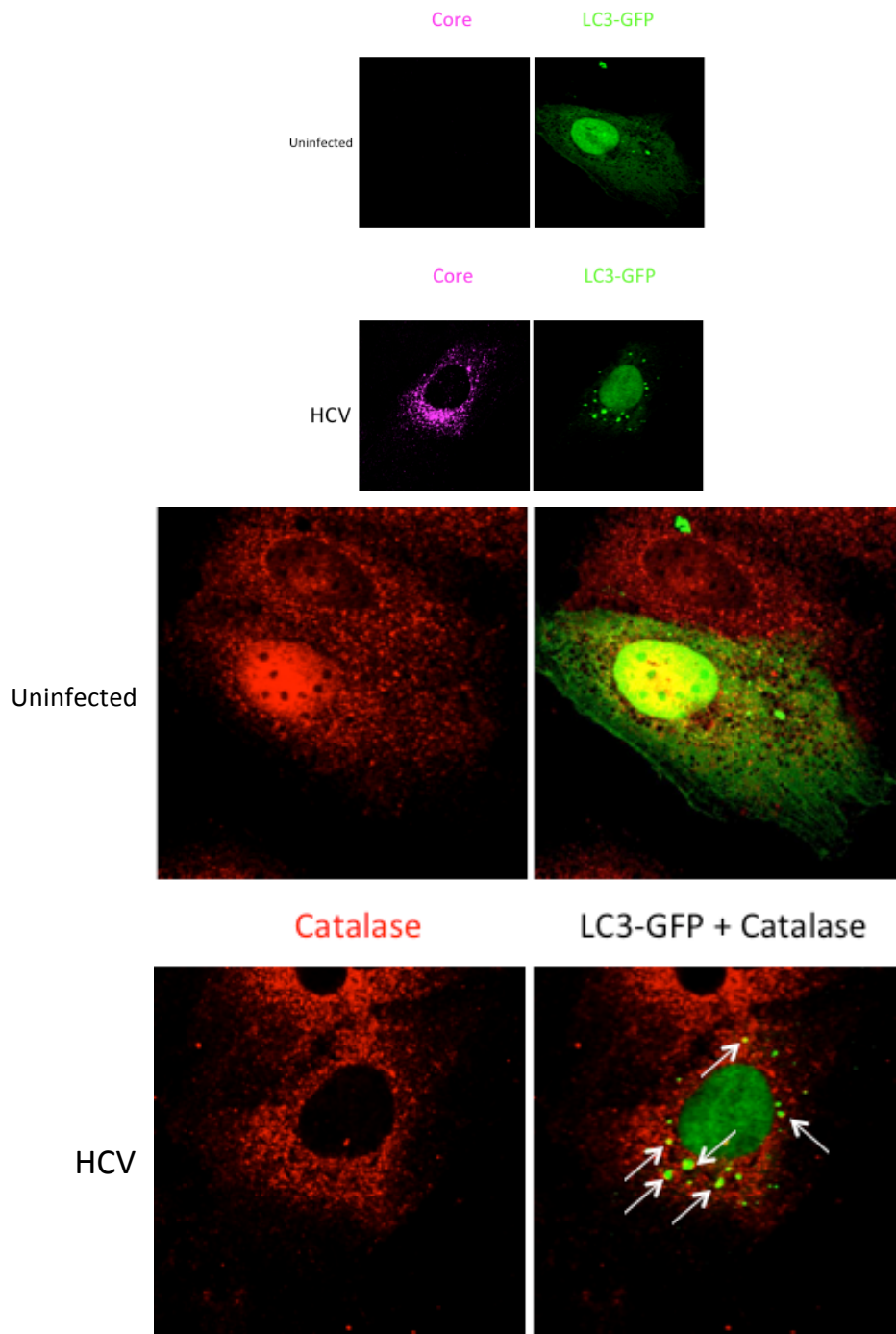


Figure 6: HCV induces Pexophagy. HCV-infected and uninfected Huh7 cells were transfected with LC3-GFP and 48h post transfection the cells were fixed and immunostained for HCV core (pink) and Catalase, a peroxisomal protein (Red). The confocal images show that in HCV infected cells catalase and LC3—GFP merge at distinct sites. Colocalization of GFP staining with catalase showed numerous yellow puncta (shown by white arrows in the LC3-GFP + Catalase merged panel signifying that peroxisomes were undergoing autophagy.

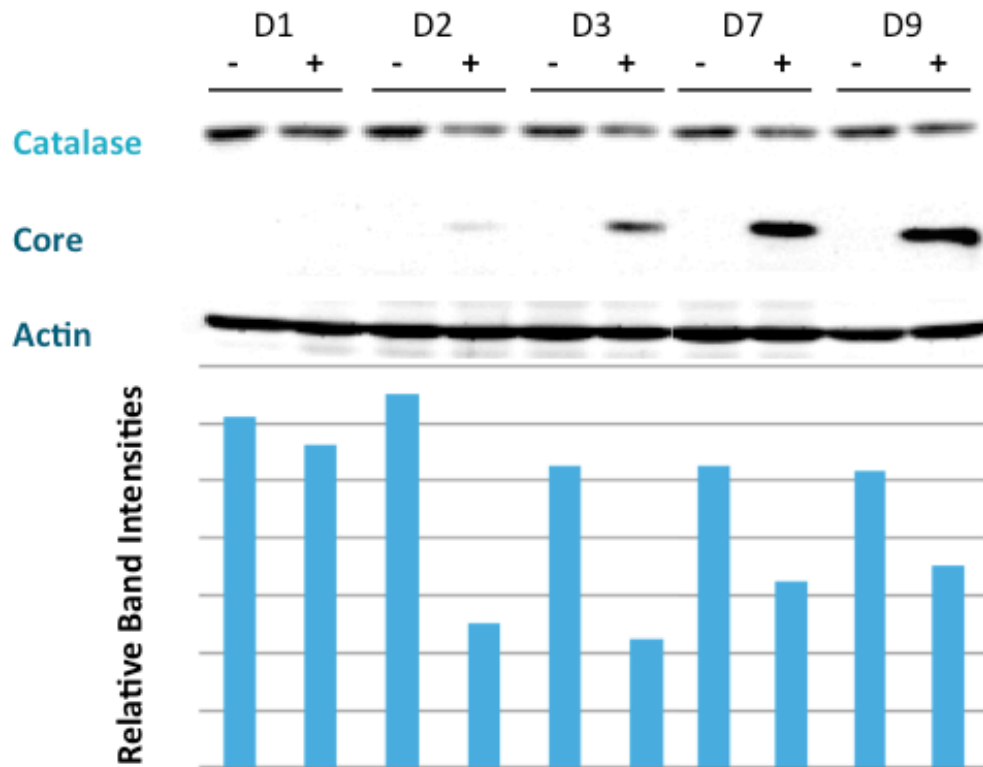


Figure 7: HCV promotes peroxisomes degradation. Western blot analysis of Catalase in HCV-infected and uninfected Huh7 cells collected at the indicated time points post infection. The Western blot reveals that as infection progresses, peroxisomal protein levels decrease. Densitometric analysis of catalase band is presented in the graphics below blots.

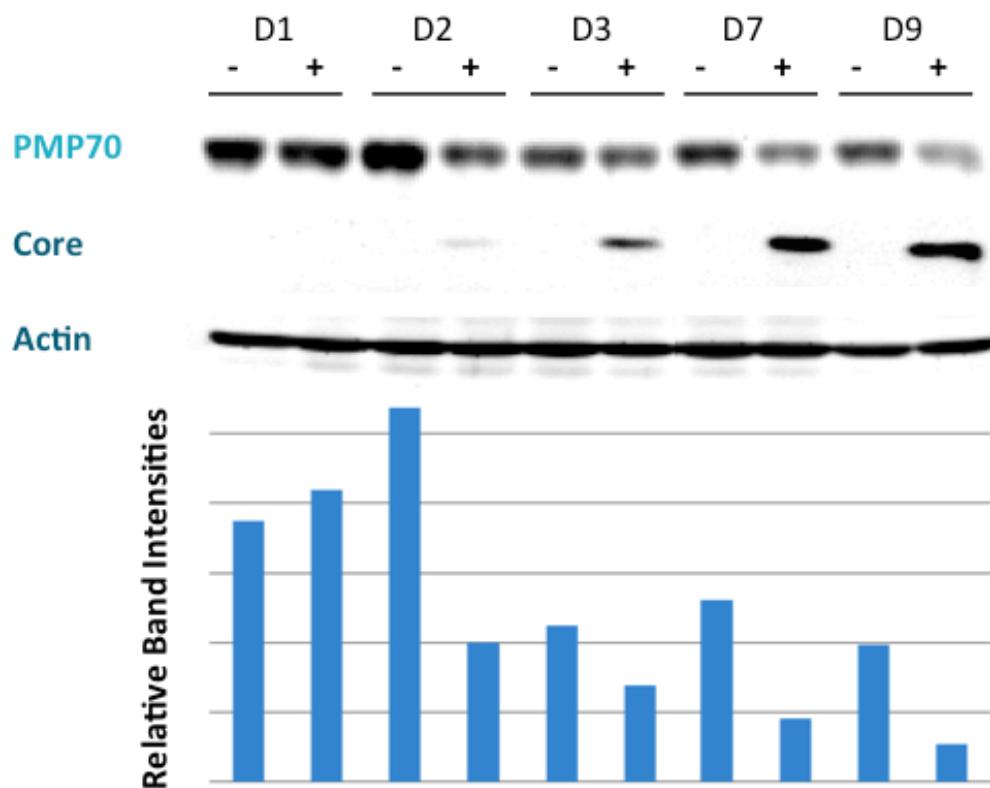


Figure 8: HCV promotes peroxisomes degradation. Western blot analysis of PMP70 in HCV-infected and uninfected Huh7 cells collected at the indicated time points post infection. The Western blot reveals that as infection progresses, peroxisomal protein levels decrease. Densitometric analysis of PMP70 band is presented as well.

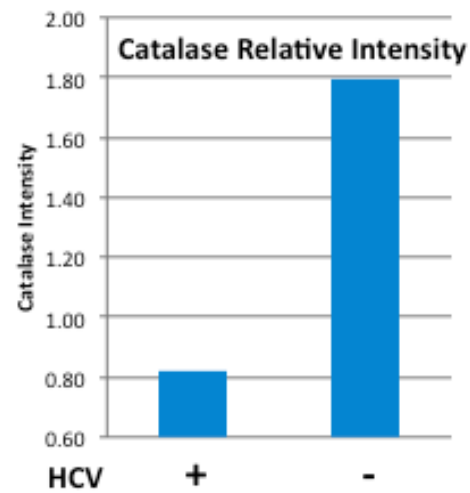
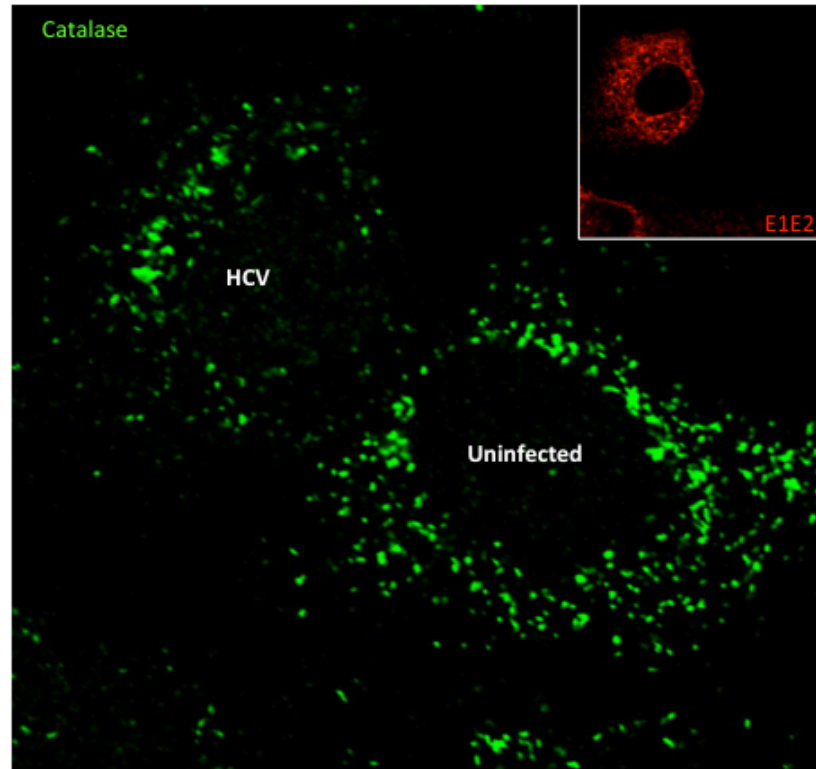


Figure 9: HCV infection promotes decline in total peroxisomes levels. HCV infected Huh7 cells were immunostained for catalase (peroxisome marker) and HCV E2 (infection marker) protein. Confocal image of catalase is shown in green and inset shows HCV E2 in red. The relative intensity of catalase staining is shown under confocal images. A significantly decreased level of peroxisomes is seen in HCV infection.

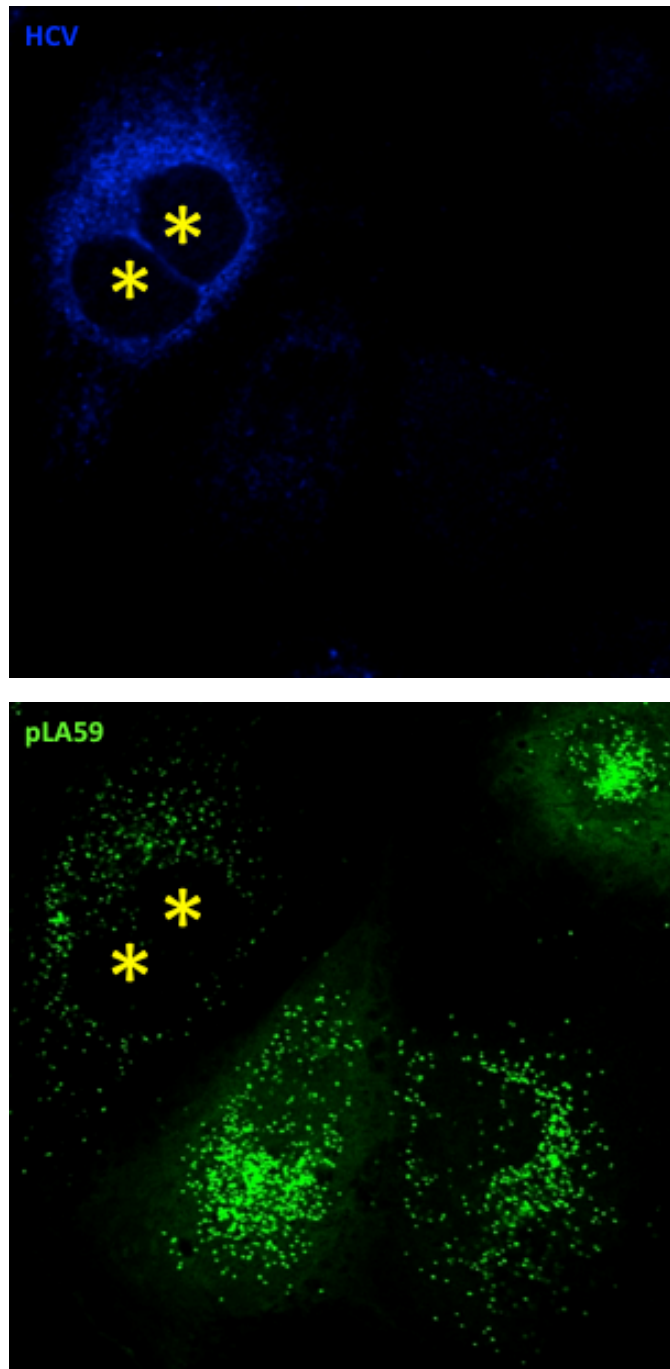


Figure 10: HCV infection promotes peroxisomes degradation. HCV-infected Huh7 cells were transfected with pLA59 plasmid, which encodes GFP-tagged peroxisomal protein and immunostained for HCV core protein. HCV core protein is shown in blue, and pLA59-GFP in green. The HCV infected cells are labeled with yellow asterisk. HCV infected cells display less GFP signal compared to uninfected cells.

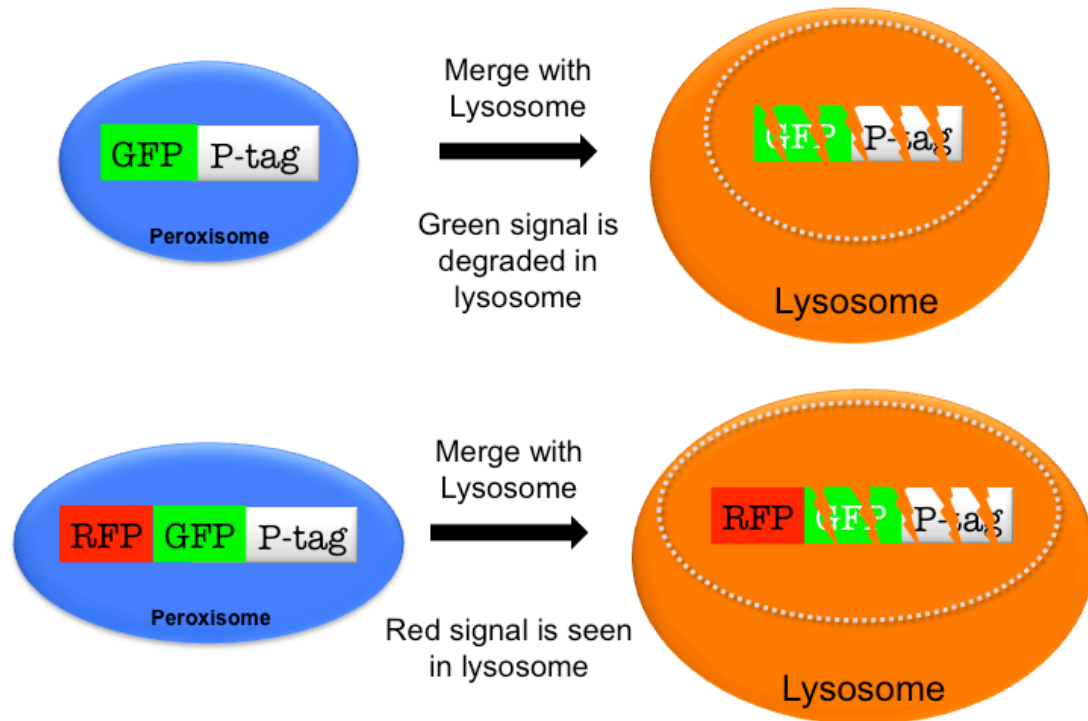


Figure 11: Differential stability of GFP and RFP in different cellular environments is used to monitor autophagic flux. GFP and RFP are both stable in the cytosolic environment of a cell, but RFP is more stable in the lysosome, while GFP will degrade, thus if the autophagosome fuses with the lysosome we can track it by noting an RFP signal. This plasmid system allows specific proteins to be tagged and then to follow their path through autophagy, i.e. if they complete it by going to the lysosome.

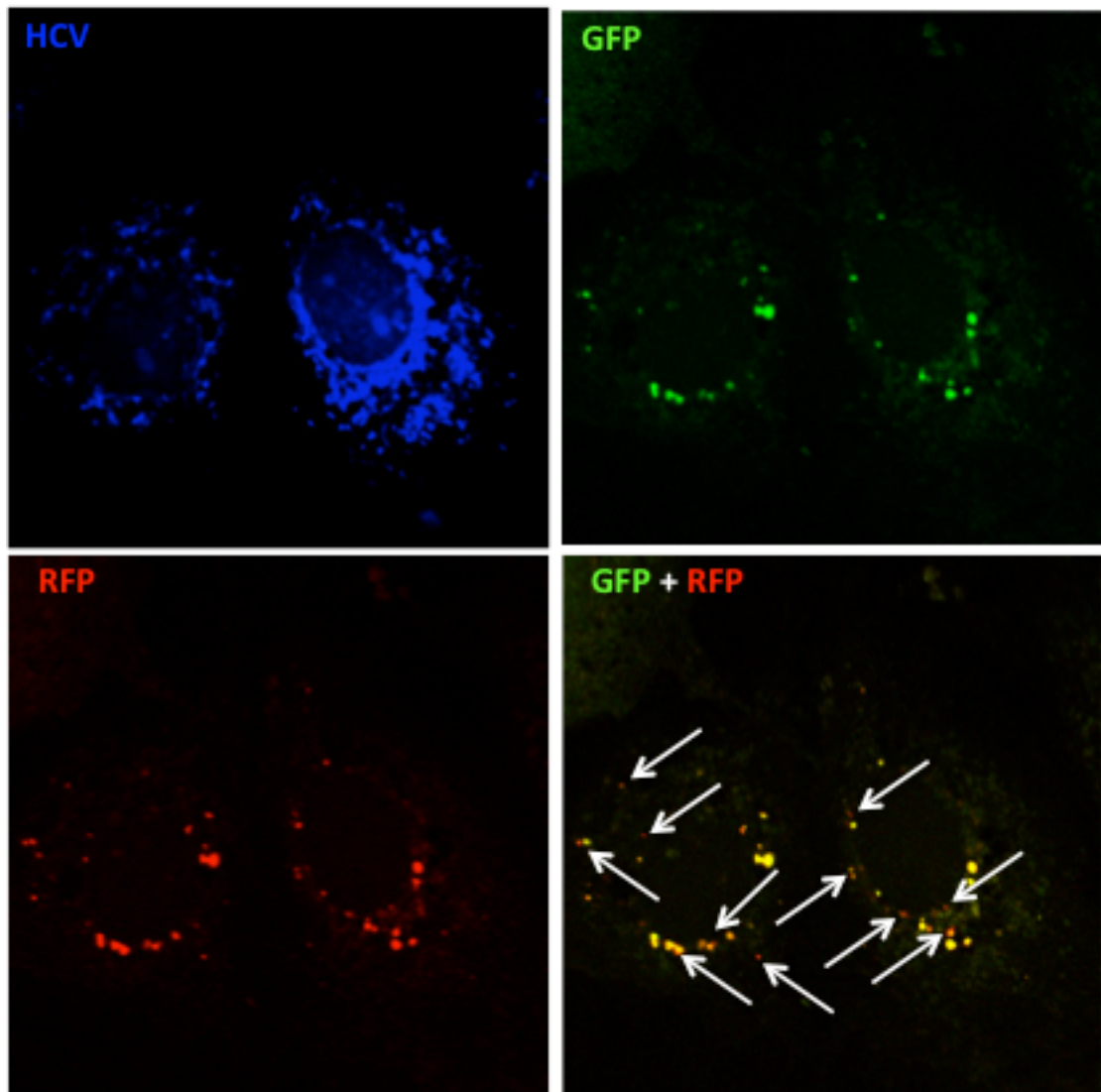


Figure 12: HCV triggers complete pexophagy. HCV-infected Huh7 cells were transfected with pTF-LC3 plasmid which encodes LC3 protein tagged to GFP and RFP. As explained in the model in Figure 11, RFP is more stable in lysosomes compared to GFP. Hence if GFP-RFP-LC3 is translocated to lysosomes more red signal is observed. HCV core staining is shown in blue. The confocal images depict distinct red spots (marked by white arrows in the GFP+RFP merged panel) suggesting that HCV triggers complete autophagy.

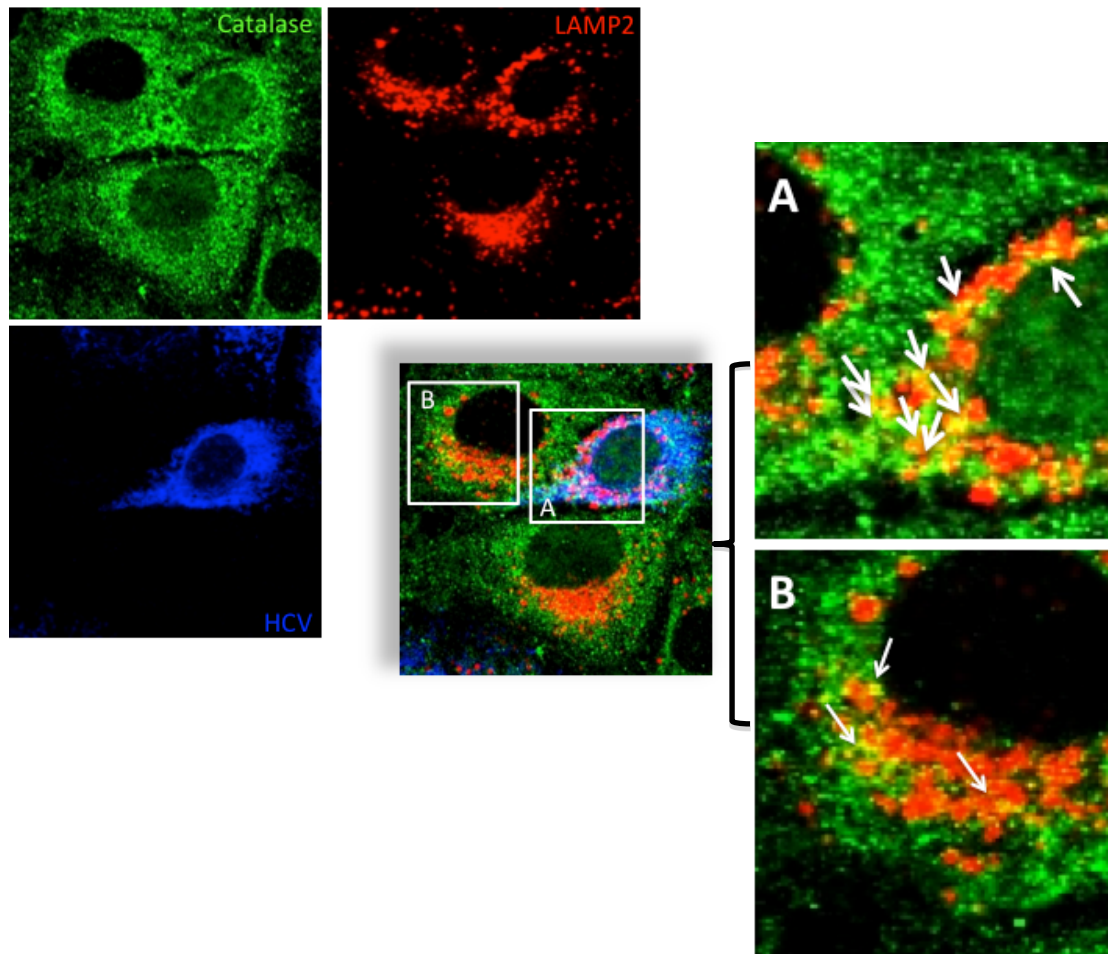


Figure 13: Higher incidence of formation of Pexophagolysosomes in HCV infected cells. HCV-infected and uninfected Huh7 cells were immunostained for Catalase (Green) and LAMP2, a lysosomal marker (Red). In infected cells (A), we see a greater colocalization of catalase and LAMP2 (see the white arrows in zoomed sections of the Red+Green merged panel) than in uninfected cells (B), suggesting that peroxisomes are going to the lysosome, undergoing pexophagy.

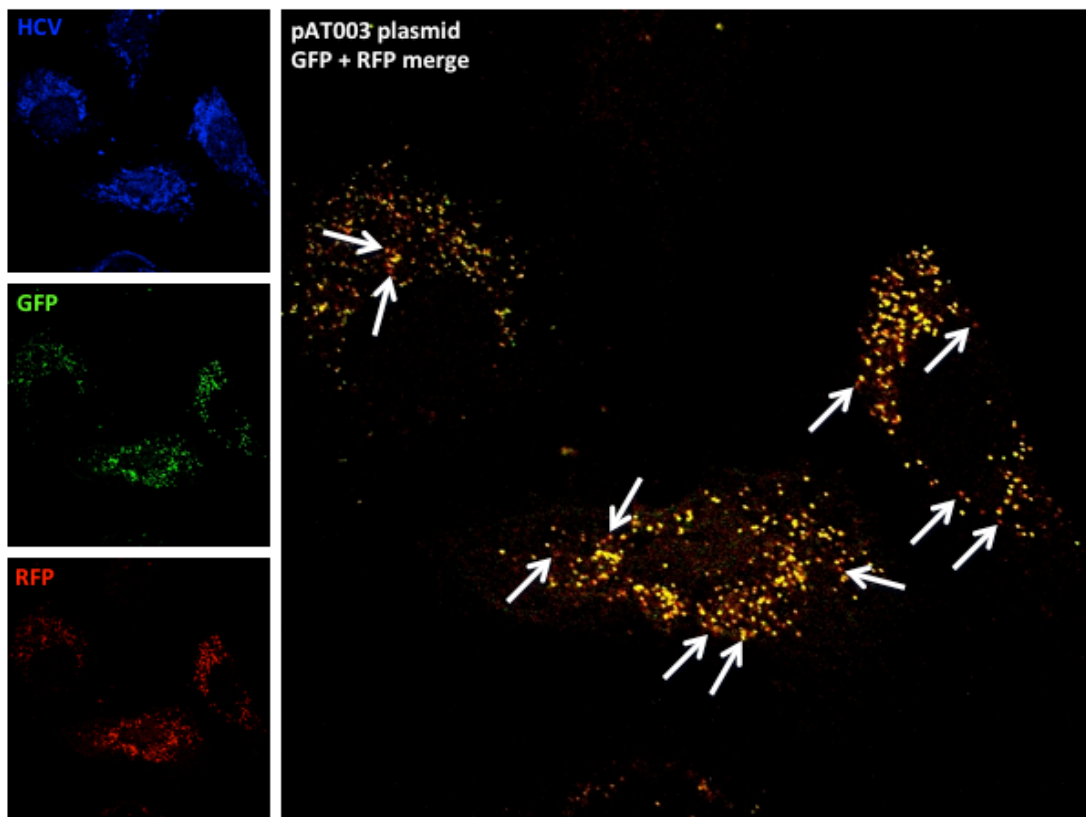


Figure 14: HCV promotes complete pexophagy. HCV-infected cells were transfected with pAT003 plasmid encoding GFP and RFP fusion protein tagged to a peroxisomal localization sequence. HCV core protein staining is shown in blue. As explained in Figure 11 RFP is more stable than GFP in the lysosomes. The merged panel shows distinct red spots (marked by white arrows) indicating that in HCV-infected cells peroxisomes are delivered to the lysosomes.

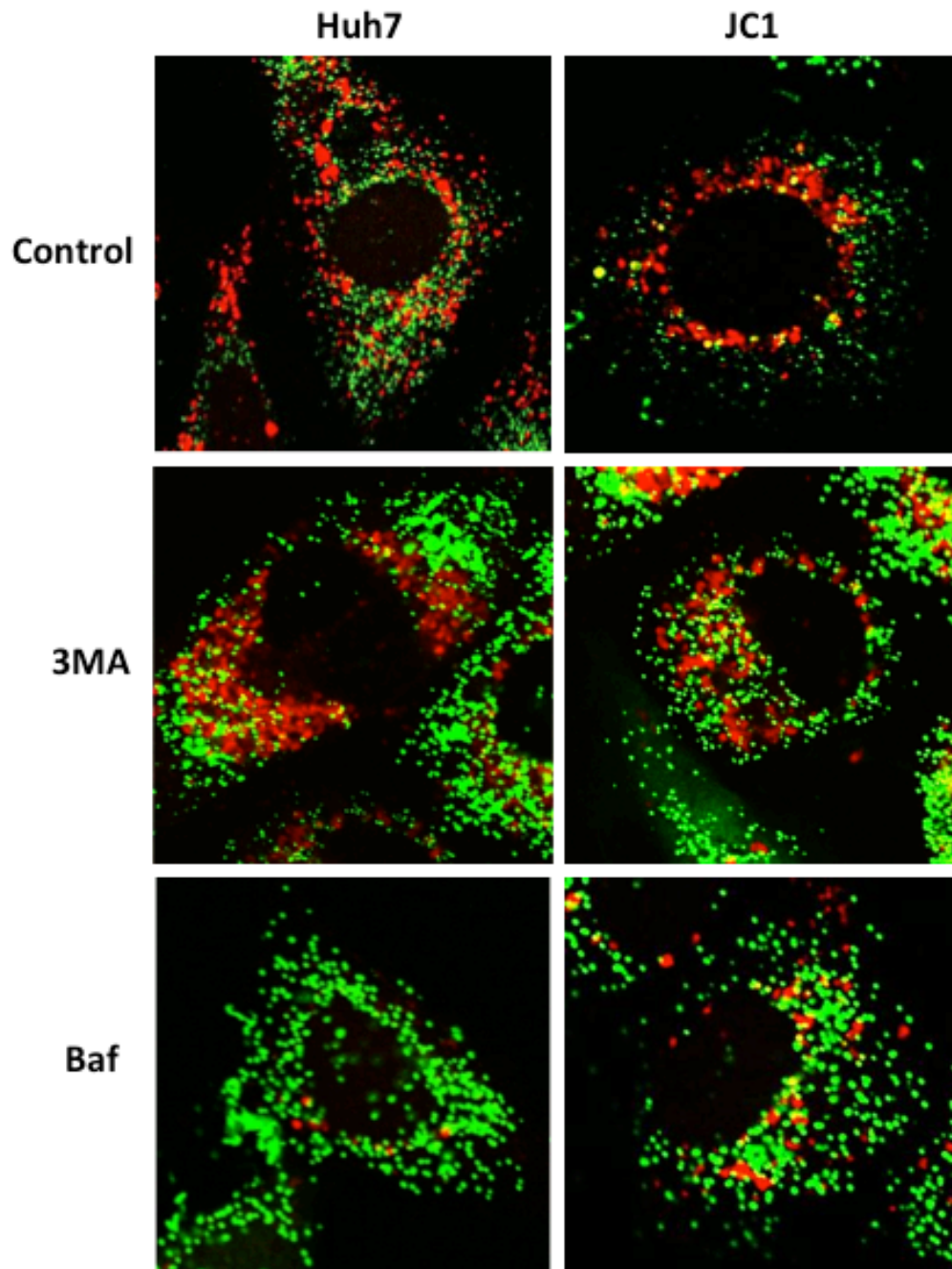


Figure 15: Treatment with autophagy inhibitors restores peroxisomes levels. HCV-infected and uninfected Huh7 cells transfected with pLA59 were treated with 3-MA and Bafilomycin (Baf) for 12h. The cells were fixed and stained for LAMP2. Confocal images display peroxisomes labeled with GFP in green and lysosomes labeled with LAMP2 in red.

In HCV-infected cells the autophagy inhibitors, 3-MA and bafilomycin, rescue peroxisomal levels to almost physiological levels.

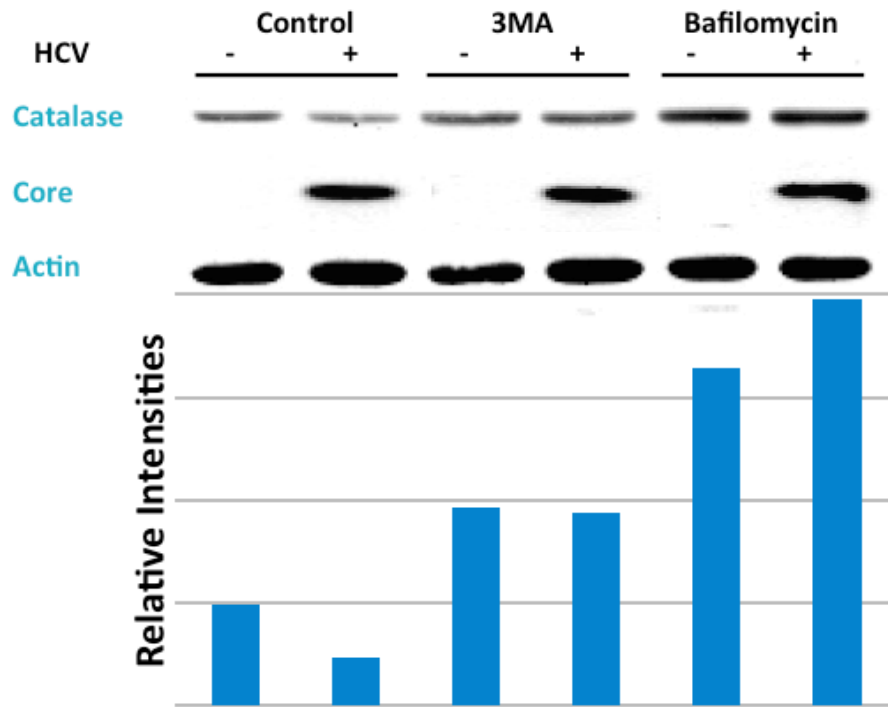


Figure 16: Treatment with autophagy inhibitors restores peroxisomes levels. Western blot analysis of catalase and PMP70 in uninfected and HCV-infected cells treated with autophagy inhibitors 3MA and Bafilomycin. HCV core is used as infection marker. Beta-actin is used as internal protein loading control.

References

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- 1 Topley, W. W. C. (William Whiteman Carlton); Wilson, Graham S. (Graham Selby); Collier, L. H. (Leslie Harold); Balows, A. (Albert); Sussman, Max.; Topley, W. W. C. (William Whiteman Carlton) (1998). *Topley Wilson's microbiology and microbial infections*. London: Arnold. p. 745.
 - 2 Zuckerman, Arie J.; Banatvala, J. E.; Pattison, J. R. (John Ridley) (1987). *Principles and practice of clinical virology*. Chichester ; New York: Wiley. p. 135. ISBN 0-471-90341-8.
 - 3 Purcell, Robert H. "Hepatitis Viruses: Changing Patterns of Human Disease." *Proc. Nati. Acad. Sci. USA* 91 (1994): 2401-406.
 - 4 "World Hepatitis Day." Centers for Disease Control and Prevention. Centers for Disease Control and Prevention, 23 July 2012. Web. 1 Sept. 2012.
 - 5 Moradpour D, Penin F, Rice CM. "Replication of hepatitis C virus." *Nat Rev Microbiology*. 2007 Jun; 5(6):453-63. Epub 2007, May 8.
 - 6 Smith, D. B., Pathirana, S., Davidson, F., Lawlor, E., Power, J., Yap, P. L. & Simmonds, P. (1997). The origin of hepatitis C virus genotypes. *J Gen Virol* 78, 321–328.
 - 7 Shepard CW, Finelli L, Alter MJ (2005) Global epidemiology of hepatitis C virus infection. *Lancet Infect Dis* 5: 558–567.
 - 8 Armstrong GL, Wasley A, Simard EP, McQuillan GM, Kuhnert WL, Alter MJ. The prevalence of hepatitis C virus infection in the United States, 1999 through 2002. *Ann Intern Med*. 2006 May 16;144(10):705–14.
 - 9 Hoofnagle, J. H. "Hepatitis C: The Clinical Spectrum of Disease." *Hepatology* 26.S3 (1997): 15S-20S.
 - 10 Hoofnagle, J. H. Course and outcome of hepatitis C. *Hepatology* (2002). 36, S21–S29.
 - 11 Timm J, Roggendorf M (2007) Sequence diversity of hepatitis C virus: implications for immune control and therapy. *World J Gastroenterol* 13: 4808–4817.
 - 12 Zhu, H., Nelson, D.R., Crawford, J.M., Liu, C. "Defective JaK-Stat Activation in Hepatoma Cells is Associated with Hepatitis C Viral INF-alpha Resistance". *J Interferon and Cytokine Res* 2005;25:528-39.

13 Thiel, H. J. et al. in *Virus taxonomy. VIIIth Report of the International Committee on Taxonomy of Viruses* (eds Fauquet, C. M., Mayo, M. A., Maniloff, J., Desselberger, U. & Ball, L. A.) 979–996 (Academic Press, 2005).

14 Chevaliez S, Pawlotsky JM. HCV Genome and Life Cycle. In: Tan SL, editor. *Hepatitis C Viruses: Genomes and Molecular Biology*. Norfolk (UK): Horizon Bioscience; 2006. Chapter 1.

15 You, S., Stump, D. D., Branch, A. D. & Rice, C. M. (2004). A cis-acting replication element in the sequence encoding the NS5B RNA-dependent RNA polymerase is required for hepatitis C virus RNA replication. *J Virol* 78, 1352–1366.

16 Thurner C, Witwer C, Hofacker IL, Stadler PF. Conserved RNA secondary structures in Flaviviridae genomes. *J Gen Virol*.2004; 85:1113–1124.

17 Han JH, Shyamala V, Richman KH, Brauer MJ, Irvine B, Urdea MS, Tekamp-Olson P, Kuo G, Choo QL, Houghton M. Characterization of the terminal regions of hepatitis C viral RNA: identification of conserved sequences in the 5' untranslated region and poly(A) tails at the 3' end. *Proc Natl Acad Sci USA*. 1991;88:1711–1715.

18 Honda M, Ping LH, Rijnbrand RC, Amphlett E, Clarke B, Rowlands D, Lemon SM. Structural requirements for initiation of translation by internal ribosome entry within genome-length hepatitis C virus RNA. *Virology*. 1996;222:31–42.

19 Rosenfeld, Amy B., Racaniello, Vincent R. “Hepatitis C Virus Internal Ribosome Entry Site-Dependent Translation in *Saccharomyces cerevisiae* Is Independent of Polypyrimidine Tract-Binding Protein, Poly(rC)-Binding Protein 2, and La Protein”. *J Virol*. 2005 August; 79(16): 10126–10137.

20 Simmonds, Peter. “Genetic Diversity and Evolution of Hepatitis C virus – 15 Years On”. *Journal of General Virology*. November 2004 vol. 85 no. 11 3173-3188

21 Miyanari, Yusuke; Atsuzawa, Kimie; Usuda, Nobuteru; Watashi, Koichi; Hishiki, Takayuki; Zayas, Margarita; Bartenschlager, Ralf; Wakita, Takaji; Hijikata, Makoto; Shimotohno, Kunitada. The Lipid Droplet is an Important Organelle For Hepatitis C Virus Production. *Nature Cell Biology*. Volume 9. Number 9. September 2007

22 Gastaminza P, Cheng G, Wieland S, Zhong J, Liao W, Chisari FV. Cellular determinants of hepatitis C virus assembly, maturation, degradation, and secretion. *J Virol* 2008;82:2120-9.

23 Miyanari Y, Atsuzawa K, Usuda N, et al. The lipid droplet is an important organelle for hepatitis C virus production. *Nat Cell Biol* 2007;9:1089-97.

-
- 24 Shavinskaya A, Boulant S, Penin F, McLauchlan J, Bartenschlager R. The lipid droplet binding domain of hepatitis C virus core protein is a major determinant for efficient virus assembly. *J Biol Chem* 2007;282:37158-69.
- 25 Feng, Bo, Pin Mei Yao, Yankun Li, Cecilia M. Devlin, Dajun Zhang, Heather P. Harding, Michele Sweeney, James X. Rong, George Kuriakose, Edward A. Fisher, Andrew R. Marks, David Ron, and Ira Tabas. "The Endoplasmic Reticulum Is the Site of Cholesterol-induced Cytotoxicity in Macrophages." *Nature Cell Biology* 5.9 (2003): 781-92.
- 26 Halliwell, Barry. "Oxygen and Nitrogen Are Pro-carcinogens. Damage to DNA by Reactive Oxygen, Chlorine and Nitrogen Species: Measurement, Mechanism and the Effects of Nutrition." *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 443.1-2 (1999): 37-52.
- 27 Yadav, D. et al. (2002) Serum and liver micronutrient antioxidants and serum oxidative stress in patients with chronic hepatitis C. *Am. J. Gastroenterol.* 97, 2634–2639
- 28 Waris G, Felmlee DJ, Negro F, Siddiqui A. Hepatitis C virus induces proteolytic cleavage of sterol regulatory element binding proteins and stimulates their phosphorylation via oxidative stress. *J Virol* 2007;81:8122-8130
- 29 Lerat H, Kammoun HL, Hainault I, Merour E, Higgs MR, Callens C, et al. Hepatitis C virus proteins induce lipogenesis and defective triglyceride secretion in transgenic mice. *J Biol Chem* 2009;284:33466-33474.
- 30 Pal, Sampa, Stephen J. Polyak, Nazneen Bano, Wan Chong Qiu, Robert L. Carithers, Margaret Shuhart, David R. Gretch, and Aditi Das. "Hepatitis C Virus Induces Oxidative Stress, DNA Damage and Modulates the DNA Repair Enzyme NEIL1." *Journal of Gastroenterology and Hepatology* 25.3 (2010): 627-34.
- 31 He, Congcong, and Daniel J. Klionsky. "Regulation Mechanisms and Signaling Pathways of Autophagy." *Annual Review of Genetics* 43.1 (2008): 090804135627089.
- 32 Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imaizumi, T., Miyagishi, M., Taira, K., Akira, S., Fujita, T. (2004) The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat. Immunol.* 5,730-737
- 33 Li, X. D., Sun, L., Seth, R. B., Pineda, G., Chen, Z. J. (2005) Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. *Proc. Natl. Acad. Sci. U. S. A.* 102, 17717-17722.

-
- 34 Seth, R. B., Sun, L., Chen, Z. J. (2006) Antiviral innate immunity pathways (review). *Cell Res.* 16,141-147
- 35 Glick, Danielle, Sandra Barth, and Kay F. Macleod. "Autophagy: Cellular and Molecular Mechanisms." *The Journal of Pathology* 221.1 (2010): 3-12.
- 36 Barth S, Glick D, Macleod KF. Autophagy: assays and artifacts. *J Pathol* 2010; DOI: 10.1002/path.2694.
- 37 Mizushima N. Autophagy: process and function. *Genes Dev* 2007; 21: 2861–2873.
- 38 Eskelinen EL. Maturation of autophagic vacuoles in mammalian cells. *Autophagy* 2005; 1: 1–10.
- 39 Gutierrez MG, Munafo DB, Beron W, Colombo MI. Rab7 is required for the normal progression of the autophagic pathway in mammalian cells. *J Cell Sci* 2004; 117: 2687–2697.
- 40 Jager S, Bucci C, Tanida I, Ueno T, Kominami E, Saftig P et al. Role for Rab7 in maturation of late autophagic vacuoles. *J Cell Sci* 2004; 117: 4837–4848.
- 41 Webb JL, Ravikumar B, Rubinsztein DC. Microtubule disruption inhibits autophagosome–lysosome fusion: implications for studying the roles of aggresomes in polyglutamine diseases. *Int J Biochem Cell Biol* 2004; 36: 2541–2550.
- 42 Tanaka Y, Guhde G, Suter A, Eskelinen EL, Hartmann D, Lullmann-Rauch R et al. Accumulation of autophagic vacuoles and cardiomyopathy in LAMP-2-deficient mice. *Nature* 2000; 406: 902–906.
- 43 Mizushima, N. "Autophagy: Process and Function." *Genes & Development* 21.22 (2007): 2861-873.
- 44 Sir, Donna, Wen-ling Chen, Jinah Choi, Takaji Wakita, T.S. Benedict Yen, and Jing-hsiung James Ou. "Induction of Incomplete Autophagic Response by Hepatitis C Virus via the Unfolded Protein Response." *Hepatology* 48.4 (2008): 1054-061.
- 45 Kim, Seong-Jun, Personal Communication. May 31, 2012.
- 46 Dixit, Evelyn, Boulant, Steeve, Zhang, Yijing, Lee, Amy S.Y., Odendall, Charlotte, Shum, Bennett, Hacohen, Nir, Chen, Zhijian J., Whelan, Sean P., Fransen, Marc, Nibert, Max L., Superti-Furga, Giulio, Kagan, Jonathan C. Peroxisomes Are Signaling Platforms for Antiviral Innate Immunity. *Cell* 141. Pgs. 668–681, May 14, 2010.

47 Gabaldon T, Snel B, van Zimmeren F, Hemrika W, Tabak H, Huynen MA. Origin and evolution of the peroxisomal proteome. *Biol Direct* 1: 8. 2006. doi:10.1186/1745-6150-1-8.

48 Camoes, F., Bonekamp, N. A., Delille, H. K., Schrader, M. Organelle dynamics and dysfunction: A closer link between peroxisomes and mitochondria. *Journal of Inherited Metabolic Disease* (2009) 32:163–180.